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Ageing, tumour necrosis factor-alpha (TNF-α) and atherosclerosis

H. BRUUNSGAARD, P. SKINHØJ, A. N. PEDERSEN*, M. SCHROLL† & B. K. PEDERSEN *Department of Infectious Diseases, H:S, Rigshospitalet, University of Copenhagen, Copenhagen, *Centre of Preventive Medicine, Glostrup University Hospital and Ministry of Food, Agriculture and Fisheries, Glostrup, and †Department of Geriatric Medicine, H:S, Bispebjerg Hospital, Bispebjerg, Denmark*

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SUMMARY

Ageing is associated with increased inflammatory activity in the blood. The purpose of this study was to investigate if age-related increased plasma levels of TNF-α were associated with atherosclerosis in a cohort of 130 humans aged 81 years. The elderly cohort had increased circulating levels of TNF-α, C-reactive protein (CRP), total cholesterol (TC), low-density lipoproteins (LDL) and a low high-density lipoprotein (HDL)/TC ratio compared with a young control group ($n = 44$). The elderly cohort was divided by tertiles into three subgroups with low, intermediate, and high levels of TNF-α, respectively. In the group with high TNF-α concentrations a significantly larger proportion had clinical diagnoses of atherosclerosis. Furthermore, weak correlations were found between TNF-α on one hand and blood concentrations of triglycerides, leucocytes, CRP and a low HDL/TC ratio on the other which are known as risk factors of atherogenesis and thromboembolic complications. No correlations were found between TNF-α, TC, LDL, or the body mass index. In conclusion, the present study shows that in a cohort of 81-year-old humans, high levels of TNF-α in the blood were associated with a high prevalence of atherosclerosis.

Keywords tumour necrosis factor-alpha ageing atherosclerosis inflammation

INTRODUCTION

Ageing is associated with increased inflammatory activity in the blood, including increased circulating levels of TNF-α [1,2], IL-6 [1,3–6], cytokine antagonists [1,7], acute-phase proteins [8,9] and neopterin [7]. Increased inflammatory activity in the elderly may reflect age-related pathological processes. Thus, atherosclerosis is an age-related inflammatory disease [10] reflected by secretion of cytokines such as TNF-α, IL-1, IL-6, and interferon-gamma (IFN-γ) and the presence of large numbers of macrophages and activated $CD4^+$ T cells within inflammatory atherosclerotic plaques [11,12].

TNF-α is a multifunctional proinflammatory cytokine which may play a part in the pathogenesis of atherosclerosis. Thus, high TNF-α levels in centenarians are associated with a low ankle-brachial arterial pressure index, indicating peripheral atherosclerosis [1]. Furthermore, atherosclerosis and increased risk of thromboembolic complications have been associated with several parameters which are related to TNF, e.g. increased circulating levels of IL-6 [13–15], acute-phase proteins such as C-reactive protein (CRP) [15–19] and fibrinogen [20,21], intercellular adhesion molecule-1 (ICAM-1) [22], leucocytes [23], and a lipid profile including increased levels of triglycerides, total cholesterol (TC), and low-density lipoproteins (LDL), decreased concentrations of high-density lipoproteins (HDL), and a low HDL/TC ratio [24–28]. Thus, TNF-α is an early mediator of the acute-phase response and involved in the production of chemokines, IL-6, and CRP as well as the recruitment of leucocytes during inflammatory reactions [29]. TNF-α is also known to induce smooth muscle proliferation and to increase adherence of leucocytes to endothelial cells by inducing the expression of cell adhesion molecules such as ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) [30]. Furthermore, TNF-α induces the expression of a wide range of cytokines, including chemokines and IL-6 by endothelial cells [31]. TNF-α also has an important role in lipid metabolism [32] by decreasing the activity of 7 α-hydroxylase [33] and lipoprotein lipase and by stimulating the liver production of triglycerides [34–36]. Type-2 diabetes and atherosclerotic cardiovascular disease have common antecedents [37] and the plasma concentration of TNF-α also predicts insulin insensitivity with advancing age [2]. In the light of this, the purpose of the present study was to investigate possible links between age-related increased plasma concentrations of TNF-α, atherosclerosis, CRP, leucocytes, and the lipid profile.

Correspondence: Helle Bruunsgaard MD, Department of Infectious Diseases M7641, Rigshospitalet, Tagensvej 20, 2200 Copenhagen N, Denmark.

E-mail: infdishb@rh.dk

SUBJECTS AND METHODS

Subjects

One-hundred and thirty humans aged 81 years (63/67 women/men) from the 1914 cohort in Glostrup, which is a longitudinal study of ageing [38], were included in the present study. No one suffered from dementia. Forty-four healthy voluntary humans of median age 25 years (range 19–31 years) constituted a young control group (19/25 women/men). Blood samples were collected in the laboratory after an overnight fast. Statistical analyses were performed with and without exclusion of elderly subjects having disorders known or suspected to influence immune function: cancer at present or previously ($n = 18$), acute or chronic inflammatory disorders ($n = 4$); intakes of systemic corticosteroids ($n = 7$), acetyl salicylic acid (> 100 mg, $n = 5$), or non-steroidal anti-inflammatory drugs ($n = 15$); low haemoglobin (< 6·5 mmol/*l*, $n = 1$), increased concentrations of leucocytes ($> 15 \times 10^9$ cells/*l*, $n = 1$), increased sedimentation rate (> 30, $n = 19$), increased blood glucose (> 10 mmol/*l*, $n = 1$), increased alkaline phosphatase (> 400 U/*l*, $n = 1$), increased alanine amino transferase (> 60 U/*l*, $n = 2$), or increased carbamide (> 15 mmol/*l*, $n = 0$). In total, 51 elderly people, but no young subjects, were excluded due to these criteria.

Atherosclerosis

Clinical manifestations of atherosclerosis were defined by one of the following diagnoses: acute myocardial infarction ($n = 9$), angina pectoris ($n = 17$), intermittent claudication ($n = 8$), aortic aneurysm ($n = 1$), stroke ($n = 4$), or transient cerebral ischaemia ($n = 3$). In total, 37 elderly people were separated by these criteria.

Body-mass index

Height and weight of the elderly group were measured in accordance with the MONICA Manual [39]. Body-mass index (BMI) was calculated as weight divided by height squared.

Circulating levels of TNF-α

TNF-α was measured in plasma supplemented with trasylol. EDTA was used as anticoagulant. Plasma was stored in −80°C until analysed by a commercially available high sensitivity ELISA kit (HSTA50; R&D Systems, Abingdon, UK). The detection limit was < 180 fg/ml. The assay measured total amount of free TNF-α plus the amount bound to soluble receptors. All samples and standards were run as duplicates and the mean of duplicates was used in the statistical analyses.

Clinical chemical tests

Standard laboratory procedures were used for analyses of lipids, CRP, and leucocytes.

Statistical analysis

Groups were compared by Student's *t*-test or by χ^2 test. With regard to CRP, the majority of subjects scored below the detection limit (48 nmol/*l*). Accordingly, a rank order analysis (Kruskal–Wallis) was used to compare groups for this parameter and subjects with scores below the detection limit was set to 48 nmol/*l*. TNF-α, triglycerides, HDL, the HDL/TC ratio and leucocytes were $\log_{10}$ transformed in the statistical analyses. Linear associations were investigated by linear regression analysis. Correlations were evaluated by Pearson's correlation analysis (*r*). One exception was the CRP data which was evaluated by Spearman's rank coefficient (r_s) due to the skewed distribution. $P < 0.05$ was considered significant.

RESULTS

The elderly cohort had increased circulating levels of TNF-α (Fig. 1), triglycerides, TC, and LDL, and a decreased HDL/TC ratio (Table 1) compared with the young group. Furthermore, a higher proportion within the elderly group showed increased levels of CRP defined as > 94 nmol/*l*: elderly group, 11% ($n = 14$) *versus* young group, 0% ($n = 0$), $\chi^2 = 5.15$, $P = 0.02$. Conclusions were the same when elderly subjects with severe medical disorders or medical intakes suspected to affect TNF-α were left out (data not shown). Furthermore, there was no difference in plasma concentrations of TNF-α when the excluded elderly people were compared with the remaining elderly subgroup ($P = 0.1$).

In order to investigate if high levels of TNF-α were associated with atherosclerosis the elderly group was divided by tertiles into three subgroups with low, intermediate, and high levels of TNF-α (Fig. 1). The two subgroups with low and intermediate TNF-α levels were pooled due to low numbers of subjects with a clinical diagnosis of atherosclerosis (TNF-low). In the elderly subgroup with the highest tertile of TNF-α levels (TNF-high) a larger proportion (19 out of 43) had a clinical diagnosis of atherosclerosis compared with the TNF-low subgroup (18 out of 87) when compared by a χ^2 test, Fig. 2a. The difference in proportions became even more pronounced when individuals with health disorders or receiving medicine known or suspected to affect TNF-α were left out (12 out of 24 *versus* 10 out of 55, Fig. 2b).

In order to investigate if TNF-α was associated with well-known risk factors of atherosclerosis the TNF-high group was compared with the TNF-low with regard to levels of lipids and leucocytes. The former group had significantly higher levels of triglycerides and leucocytes and lower HDL/TC ratios, whereas there was no difference with regard to levels of HDL, LDL, TC, and CRP (Table 1). Furthermore, weak associations were found

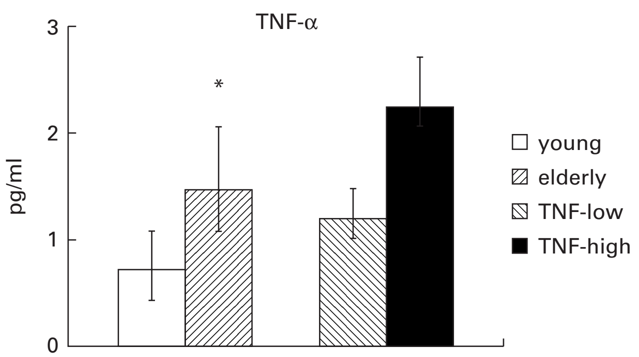

Fig. 1. Plasma concentrations of TNF-α in young and elderly humans. Young, $n = 44$; elderly, $n = 130$. The elderly group was divided by tertiles into subgroups with high, intermediate and low plasma levels of TNF-α, respectively. The latter two subgroups were pooled. TNF-high, elderly people with the highest plasma concentrations of TNF-α, $n = 43$; TNF-low, elderly people with low/intermediate plasma concentrations of TNF-α, $n = 87$. Medians and quartiles are shown. *Significant difference ($P < 0.05$) from the young controls.

Table 1. Lipids and leucocytes in young and elderly humans

	Young	Elderly	P	TNF-high	TNF-low	P
Triglycerides, mmol/*l*	0·86 (0·67–1·08) $n = 44$	1·19 (0·88–1·62) $n = 128$	< 0·0005	1·27 (0·99–1·91) $n = 42$	1·15 (0·86–1·54) $n = 86$	0·02
Total cholesterol (TC), mmol/*l*	4·5 (4·1–5·1) $n = 44$	6·4 (5·6–7·0) $n = 130$	< 0·0005	6·5 (5·6–7·2) $n = 43$	6·4 (5·8–6·9) $n = 87$	0·4
HDL, mmol/*l*	1·4 (1·2–1·8) $n = 44$	1·4 (1·1–1·7) $n = 129$	0·3	1·3 (1·0–1·8) $n = 43$	1·4 (1·2–1·7) $n = 86$	0·1
HDL/TC	0·32 (0·25–0·38) $n = 44$	0·22 (0·19–0·27) $n = 129$	< 0·0005	0·20 (0·16–0·26) $n = 43$	0·23 (0·20–0·28) $n = 86$	0·03
LDL, mmol/*l*	2·8 (2·2–3·2) $n = 44$	4·3 (3·7–4·8) $n = 128$	< 0·0005	4·2 (3·7–4·8) $n = 42$	4·3 (3·7–4·8) $n = 86$	0·9
Leucocytes, 10^9/L	5·1 (4·2–5·7) $n = 43$	5·8 (4·8–6·5) $n = 129$	0·02	6·1 (5·3–7·2) $n = 42$	5·5 (4·6–6·2) $n = 87$	0·01
CRP, mmol/*l*	< 48 (< 48–53) $n = 44$	< 48 (< 48–280) $n = 130$	0·002	< 48 (< 48–280) $n = 44$	< 48 (< 48–183) $n = 130$	0·4

Medians and quartiles are shown. Medians and range are shown for C-reactive protein (CRP). The elderly group was divided by tertiles into subgroups with high, intermediate and low plasma levels of TNF-α, respectively. The latter two subgroups were pooled. TNF-high, elderly people with the highest plasma concentrations of TNF-α; TNF-low, elderly people with low/intermediate plasma concentrations of TNF-α.

LDL, Low-density lipoprotein; HDL, high-density lipoprotein.

between TNF-α on one hand and serum concentrations of triglycerides, the HDL/TC ratio, and the leucocyte count on the other. Two cases were outliers due to large leverages. These points were left out in the analyses shown in Table 2. However, if these cases were included the linear associations showed higher P values, and furthermore, a significant correlation was found between TNF-α and HDL (data not shown). Accordingly, we thought it was more correct to leave these two points out due to their large influences on the correlation coefficients (large Cooks distance measures). TNF-α was also positively correlated with CRP in a Spearman rank order correlation analysis ($r_s = 0{\cdot}18$, $n = 130$, $P = 0{\cdot}04$). No linear associations were found between TNF-α and TC or LDL (data not shown). Furthermore, no association was found between TNF-α and BMI (data not shown).

DISCUSSION

The major findings in the present study were that 81-year-old humans showed increased plasma concentrations of TNF-α compared with young controls. High circulating levels of TNF-α in the elderly group were associated with an increased risk of a clinical diagnosis of atherosclerosis. The conclusion was the same whether elderly subjects with severe health disorders were excluded or not. Furthermore, high plasma levels of TNF-α was weakly associated with risk factors of atherogenesis and thromboembolic complications, including high levels of triglycerides, low HDL/TC ratio, CRP, and high leucocyte counts. Thus, data in the present study support the hypothesis that increased plasma concentrations of TNF-α in elderly humans are associated with atherosclerosis.

It cannot be concluded from the present cross-sectional design whether TNF-α is a causative factor in atherosclerosis, if it reflects the intrinsic inflammation within arterial lesions, or if TNF-α and atherosclerosis are independently related to a third unknown factor. Consistent with the present study, patients with peripheral vascular disease and survivors of myocardial infarction showed higher levels of TNF-α compared with age- and sex-matched controls [40,41]. In support of the hypothesis that TNF-α reflects ongoing inflammation in arterial lesions, TNF production was increased in supernatants from blood vessels from old mice compared with young mice [42]. However, raised TNF-α concentrations may also reflect inflammation elsewhere in the body promoting atherogenesis. In support of this hypothesis, aspirin reduced the risk of first myocardial infarction by a mechanism that involves a decrease in the production of CRP [16]. Furthermore, increased levels of CRP were strongly associated with coronary heart disease as well as with minor chronic insults such as smoking, symptoms of chronic bronchitis, *Helicobacter pylori* and *Chlamydia pneumoniae* infections in elderly people from general practices [19]. It is commonly accepted that TNF-α and IL-1β

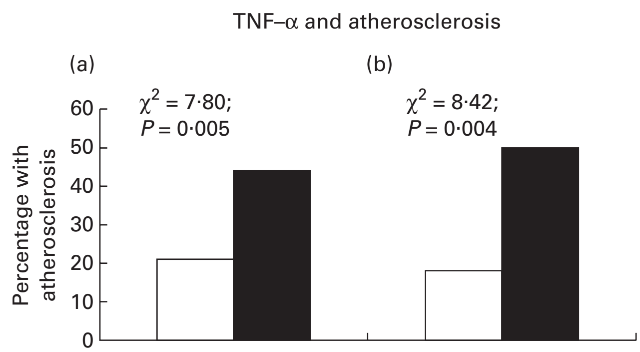

Fig. 2. Proportions of elderly subjects with a clinical diagnosis of atherosclerosis within subgroups having high *versus* low/intermediate plasma concentrations of TNF-α. The elderly group was divided by tertiles into subgroups with high, intermediate and low plasma levels of TNF-α, respectively. The latter two subgroups were pooled. TNF-high, elderly people with the highest plasma concentrations of TNF-α (■); TNF-low, elderly people with low/intermediate plasma concentrations of TNF-α (□). (a) The whole elderly group: TNF-high, $n = 43$; TNF-low, $n = 87$. (b) Elderly subjects with health disorders or receiving medicine known or suspected to affect TNF-α are left out: TNF-high, $n = 24$; TNF-low, $n = 55$.

Table 2. Linear regression models of lipids and leucocytes on TNF-α in 81-year-old people

Dependent variable	Independent variable	Coefficient	s.e.m.	P	r
Log_{10} (triglycerides) $n = 126$	Log_{10} (TNF-α)	0·16	0·081	0·05	0·18
Log_{10} (HDL/TC) $n = 126$	Log_{10} (TNF-α)	−0·28	0·12	0·03	0·2
Log_{10} (leucocytes) $n = 127$	Log_{10} (TNF-α)	0·11	0·048	0·02	0·2

TC, Total cholesterol; HDL, high-density lipoprotein; r, Pearson's correlation coefficient.

together with IL-6 induce liver production of CRP [29]. In accordance with this, TNF-α was positively correlated with CRP (rank order correlation) in the present study. However, one weakness of the study was that the CRP assay was not very sensitive for low levels. Thus, the majority of both elderly and young subjects showed CRP levels below the detection level of the assay. Furthermore, atherosclerosis was only associated with marginal elevations in plasma concentrations of TNF-α. This is in accordance with other reports of only marginal elevations in circulating concentrations of IL-6, sTNFR, and CRP in relation to atherosclerosis [15,19,40], and it may reflect chronic low-grade inflammation. Monocytes are the principal TNF-α-producing cells in the blood. In support of the present data, the neopterin level, which reflects monocyte activation, was correlated with the score of atherosclerotic lesions [43].

With regard to the association between TNF-α and lipids, fenofibrate treatment lowered plasma TNF-α as well as lipids in patients with atherosclerosis [44]. Furthermore, others have reported associations between CRP and the lipid profile in the blood [19]. TNF-α was only weakly related to high levels of triglycerides and a low HDL/TC ratio in the present study. Accordingly, it is difficult to make any interpretation of the clinical effect, which may be marginal. However, TNF-α is known to increase serum concentrations of triglycerides by decreasing adipose tissue lipoprotein lipase activity, resulting in increased levels of free fatty acids, and by stimulating the liver production of triglycerides [34–36]. Furthermore, TNF-α decreases the concentration of HDL [45,46]. However, HDL has also been shown to decrease TNF-α production from monocytes [47] and to inhibit TNF-α induction of VCAM-1 and E-selectin by endothelial cells [48], and increased plasma concentrations of TNF-α were induced after stimulation of monocytes and macrophages within the atherosclerotic plaques by oxidized LDL [12,49,50].

It is commonly accepted that TNF-α is involved in the physiological and metabolic abnormalities found in cachectic states [51,52]. However, TNF-α may also play a role in obesity [52], e.g. plasma levels of TNF-α were positively correlated with body fat in a study of humans with a wide age range [2]. It has been suggested that TNF-α may have a mechanistic role in the control of body mass in normal weight-controlled situations, and that abnormalities in either its production (during cachexia) or action (during obesity) are responsible for the lack of control of body weight [52]. We did not detect any association between BMI and plasma levels of TNF-α in the present study. This may reflect that TNF-α is not associated with body weight as such. The lack of a relationship between TNF-α and BMI is consistent with reports of no association between plasma TNF-α and weight loss among 127 nursing home patients aged 60–80 years [53] and no linear correlations between BMI and plasma levels of TNF-α in elderly underweight anorectic patients [54] or in a large cohort of centenarians [1]. Further studies focusing on body composition and TNF in the elderly are warranted.

The elderly cohort in the present study represents an approximation to a normal population, showing a wide spectrum of 'physiological' ageing but with the same chronological age. One major weakness of the present study is that when the elderly group was compared with the young controls the effect of age and atherosclerosis could not be separated. However, it is questionable if the process of ageing can be separated from atherosclerosis because the extent of atherosclerosis increases strongly with age [10]. Thus, subjects with severe manifestations of atherosclerosis may have suffered most from the process of ageing when compared with subjects of the same chronological age. Furthermore, the effect of age was bypassed in the present study when associations between TNF-α and atherosclerotic manifestations were evaluated within the elderly group due to the narrow age range in this group. Exclusions of subjects with severe medical diseases or intake of medicine known to affect levels of TNF-α did not influence the conclusions of the study, indicating that other severe health disorders were not responsible for the increased levels of TNF-α in the elderly group.

In conclusion, the present results show that in a cohort of 81-year-old humans high inflammatory activity was associated with increased prevalence of atherosclerosis. Further research is needed to understand if TNF-α reflects the degree of atherosclerosis, or if TNF-α is an age-related causative factor. The answer is important for future intervention strategies.

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